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Supplemental information

Apoferritin improves motor deficits in MPTP-treated mice by regulating brain iron metabolism and ferroptosis

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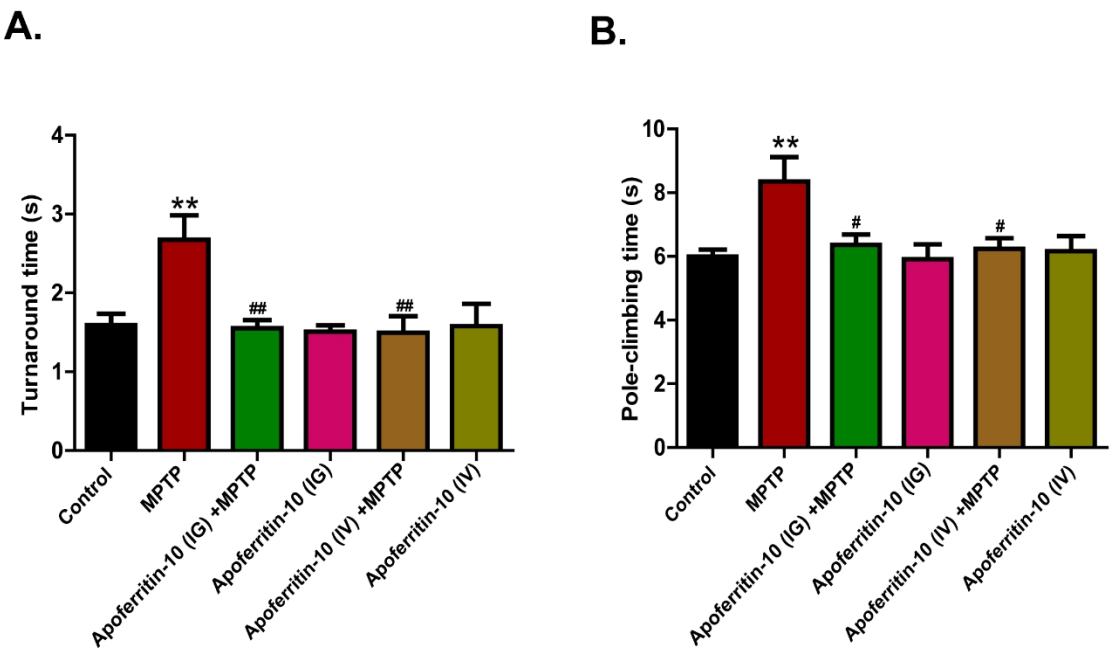
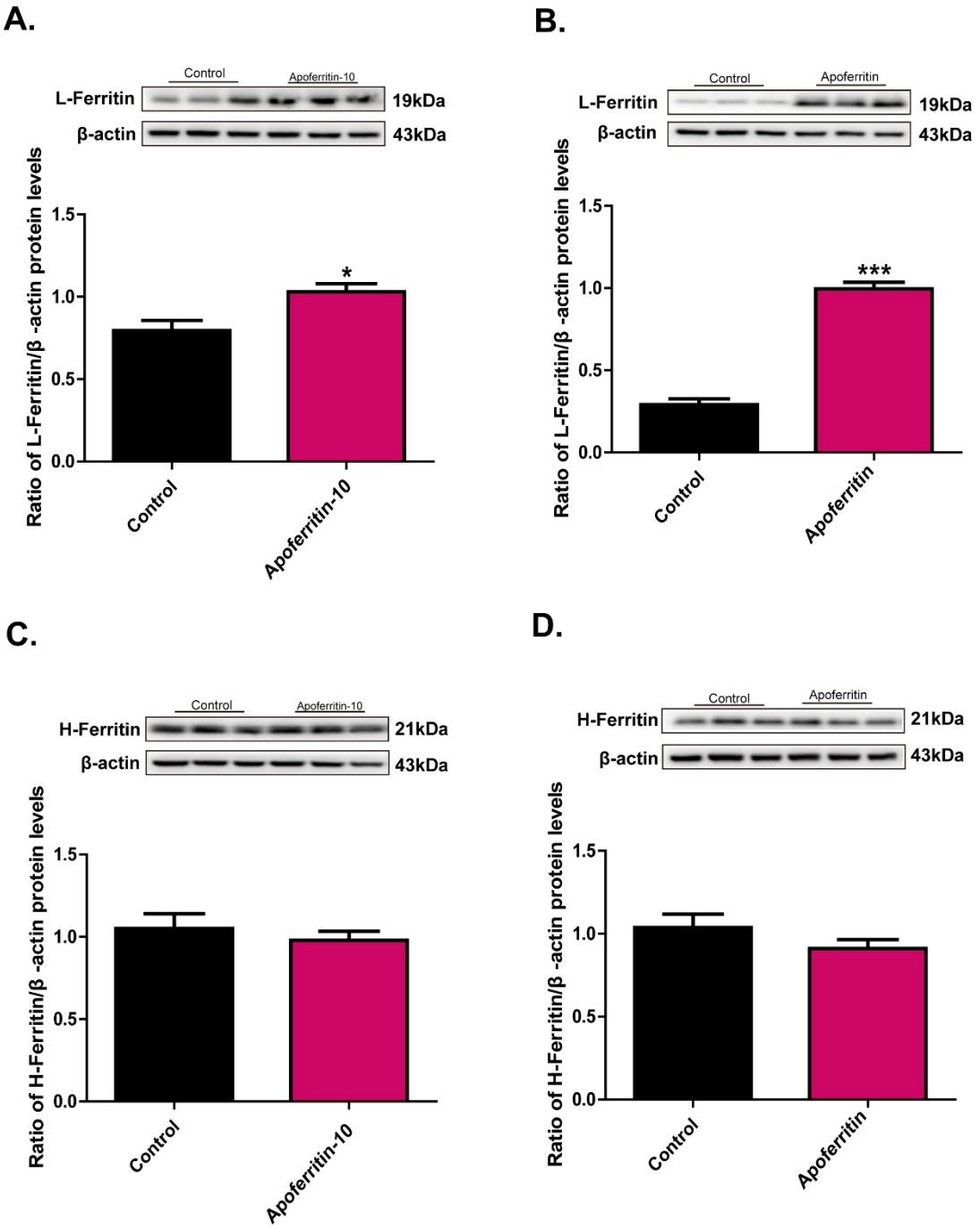
Supplemental figures and legends



Supplementary Figure 1. Intragastric Gavage or Intravenous Injection of Apoferritin Improved Motor Deficits of MPTP-treated Mice, Related to Figure 1

MPTP resulted in significant motor deficits, which were inhibited by apoferritin given by intragastric gavage (IG) or intravenous (IV) injection. A. Turnaround time; B. Pole-climbing time. $^{**}P < 0.01$, compared with the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the MPTP. Data were expressed as mean ± SEM (n = 5–11 in each group).



Supplementary Figure 2. The Expression of Ferritin after Apoferritin Treatment in vivo and in vitro, Related to Figure 4

The expression of L-ferritin increased significantly after apoferritin treatment, compared with the control.

A. The expression of L-ferritin in C57BL/6J mice treated with 10 mg/kg apoferritin;

B. The expression of L-ferritin in the primary VM neurons treated with 50 ug/mL apoferritin;

C. The expression of H-ferritin in C57BL/6J mice treated with 10 mg/kg apoferritin;

D. The expression of H-ferritin in the primary VM neurons treated with 50 ug/mL apoferritin.

$^{*}P < 0.05$, $^{***}P < 0.001$, compared with the control. Data was expressed as mean ± SEM (n = 6–12 in each group).

Supplementary Table 1. Key Antibodies Used in the Experiments, Related to Figure 2-5

REAGENT	WORKING DILUTION	SOURCE	IDENTIFIER
TH	1:3000/1:1000	United States Millipore	Cat# AB152; RRID: AB_390204
GPX4	1:10000	United States Abcam	Cat# ab125066; RRID: AB_10973901
FSP1	1:1000	United States Millipore	Cat# 07-2274; RRID: AB_10807552
ACSL4	1:1000	United States Santa Cruz	Cat# sc-365230; RRID: AB_10843105
β-actin	1:10000	China Bioss	Cat#bs0061R; RRID: AB_0855480
DMT1	1:1000	United States OriGene	Cat# TA324527; RRID: AB_2758340
TfR1	1:1000	United States Abcam	Cat#ab84036; RRID: AB_10673794
Iba-1	1:200	United States CST	Cat# 17198; RRID: AB_2820254
H-ferritin	1:1000	United States Abcam	Cat#ab183781
L-ferritin	1:1000	United States Abcam	Cat#ab69090; RRID: AB_1523609
Goat Anti-Rabbit IgG-Alexa Fluor 488	1:500	China Absin	Cat#abs20025
Goat Anti-Rabbit IgG-Alexa Fluor 594	1:500	China Absin	Cat#abs20021
Goat Anti-Rabbit IgG	1:10000	China Absin	Cat#abs20011
Goat Anti-Mouse IgG	1:10000	China Absin	Cat#abs20012

Transparent Methods

Method details

Chemicals

Apoferritin from horse spleen, MPTP and tyrosine hydroxylase (TH) were purchased from Sigma (St. Louis, MO, USA). The primary antibodies of glutathione peroxidase 4 (GPX4), transferrin receptor 1 (TfR1), H-ferritin and L-ferritin were from Abcam (Cambridge, MA, USA). The primary antibody of ferroptosis suppressor protein 1 (FSP1) was from MilliporeSigma (Billerica, MA, USA). The primary antibody of long-chain acyl-CoA synthetase 4 (ACSL4) was from Santa Cruz Biotechnology (CA, USA). The primary antibody of DMT1 was from OriGene Technology (Maryland, USA). The monoclonal β-actin antibody was from Bioss (Beijing, China). The goat anti-rabbit IgG labeled with HRP and goat anti-mouse IgG labeled with HRP were from absin (Shanghai, China). ECL ultrasensitive chemiluminescence kit was from MilliporeSigma (Billerica, MA, USA). Other biological reagents and materials are from local commercial sources.

Primary Cultured Ventral Mesencephalon (VM) Neurons

Primary cultures of VM neurons were obtained from embryonic 14-day Sprague–Dawley rat. Briefly, VM was dissected from the embryonic rat brain under the dissection microscope and then mechanically dissociated with a pipette until the tissue was dispersed. After centrifugation, cells were suspended in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin and seeded on poly-d-lysine-coated 12-well culture plates. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C for 18 h, and then the culture medium was changed to serum free DMEM/F12 supplemented with 2% B27. Cells were grown for a further 4 days before use. For experiments, VM neurons were treated with 50 μg/ml Apoferritin for 24 h.

Animal Treatments

The C57BL/6J male mice used in this study were all from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The mice were housed one animal per cage with food pellets and water available ad libitum. The room was maintained at a constant temperature and humidity on a 12 h light/dark cycle. The mice were adapted to the laboratory environment for 1 week before the experiments. The Animal Ethics and Experimentation Committee of the Qingdao University approved the use of animals for this study.

MPTP is diluted with normal saline (NS) to 6 mg/ml, and the injection volume is 30 mg/kg; Apoferritin is diluted with NS to 2 mg/ml stock solution. The working concentration is 10 mg/kg or 15 mg/kg. Male C57BL/6J mice aged 9-10 weeks were randomly divided into four groups: control group (normal saline group), MPTP group, apoferritin+MPTP group, and apoferritin group. Pre-protection was given for 3 days before the experiments (Mice in the control group and MPTP group were given NS by intragastric gavage; Mice in apoferritin+MPTP group and apoferritin group were given apoferritin by intragastric gavage (IG) or intravenous (IV)

injection). On the 4th day after apoferritin treatment, MPTP was given intraperitoneally for 5 consecutive days to induce PD models (control group and apoferritin group intraperitoneally injected NS；MPTP group and apoferritin+MPTP group intraperitoneally injected MPTP).

Open-field Test

The open-field test was used to assess the general behavior and locomotor activity. Mice were gently placed in the center of a dedicated black box (40 cm×40 cm×40 cm). Locomotor behavior was video-recorded for 10 min by a computer for automatic analysis and the total movement distance of each animal was recorded.

Pole-climbing Test

A self-made straight wooden pole with a diameter of 1.2 cm and a height of 50 cm was used to do the pole-climbing test. A small wooden ball is placed on the top of the pole and wrapped with gauze to prevent mice from slipping. Mice were habituated to the pole on the day before testing. And then the animals were recorded via digital video on the next day. The amounts of time for the mouse to turn towards the ground (time to orient down) and to reach the ground (time to descend) were recorded. The average scores for each mouse were determined based on five tests.

Perls' Iron Staining

After the mice were perfused with NS and 4% paraformaldehyde (PFA). The brains were taken and fixed in 4% PFA for 4-6 hours, and then immersed in 20% and 30% sucrose for sugar precipitation. Brain blocks containing the SN were sectioned coronally at 20 μm on a freezing microtome and stored at -20℃. Perls' staining was utilized to detect the presence of iron in brain sections by a complex hydrated ferric ferrocyanide substance as described previously (Jiang et al., 2010). Sections were fixed with 4% PFA for 5 minutes and washed with ddH_2O for 30 seconds (not overtime). Sections were immersed for in a ready-to-use iron staining solution (2% HCL-potassium ferrocyanide), followed by three washes with PBS. Negative control sections were prepared in which the HCl and potassium ferrocyanide solutions were omitted. The sections were then immersed in 99% methanol and 1% hydrogen peroxide for 20 min to eliminate endogenous peroxidase activity. The DAB reaction product was observed under an Olympus microscope and the images were captured by a video camera (OLYMPUS, Japan) at a final magnification of 200×.

Immunofluorescence

For immunofluorescence, the sections of SN were stained with TH antibody. After three washes with 0.01% phosphate-buffered saline (PBS, pH 7.4) for 10 min, sections were incubated in 5% donkey serum-PBS for 1 h at room temperature and then incubated overnight with the primary antibody of TH (1:1000) or Iba-1(1:200). After washing, secondary antibody of Alexa Fluor ® 488 donkey anti-rabbit IgG was applied to sections for 2 h at room temperature.

Nuclei were stained with DAPI at room temperature for 10 min in the dark and washed twice with PBS. Then sections were mounted with 70% glycerin and examined using digital pathology section system (OLYMPUS, Japan). The same anatomical landmarks were used to select three sections through the SN from each mouse to count TH-positive cells at a final magnification of 400×. Values represent the mean TH-positive cells from each section.

Western Blotting

SN tissues were dissected from the brain of mice and lysed with lysis buffer on ice for 30 min. The harvested lysates were centrifuged at 12,000×g for 20 min at 4 °C, and the supernatants were used for analysis. Protein concentration was determined by a BCA protein assay kit (CWBIO China). Proteins with 5 × loading buffer (Beyotime) were incubated at 100°C for 5 min. The total 20 μg protein was separated by 8-12% SDS polyacrylamide gels and the protein in the gel was transferred to a PVDF membrane (Millipore, MA, USA). After overnight blocking with TBST containing 5% non-fat milk or BSA for 2 hours at room temperature, membranes were incubated overnight at 4 °C with the primary antibodies against TH (1:3000), DMT1 (1:800), TfR1 (1:1000), H-ferritin (1:1000), L-ferritin (1:1000), ACSL4 (1:1000), GPX4 (1:10000), FSP1 (1:1000), β-actin (1:10000). Goat anti-rabbit or goat anti-mouse IgG labeled with HRP (Santa Cruz Biotechnology, Texas, USA) was used at 1:10,000 and incubated with the membranes for 1 h at room temperature. Cross-reactivity was visualized using ECL western blotting detection reagents (Millipore, USA) and then was analyzed through scanning densitometry by a UVP BioDoc-It Imaging System (UVP, Upland, USA).

GSH/GSSG Ratio Detection

The total reduced GSH and GSSG level were detected by GSH/GSSG assay kit (Abcam, USA) according to the manufacturer's instructions. 400 μl 0.5% NP-40 (Solarbio, China) was added to 20 mg tissue to grind the tissue thoroughly, and centrifuged at 4°C, 12000 rpm for 15 min. Extract the supernatant into a new Ep tube, then add 1 volume of TCA (Abcam, USA) to 5 volume of the sample and vortex to mix. After incubation on ice for 5-10 min and centrifugation at 4°C, 12000 g for 5 min. The supernatant was used to detect the level of GSH/GSSG by GSH/GSSG assay kit. The signal was read by a fluorescence microplate reader at Ex/Em = 490/520 nm.

Statistical Analysis

The results were analyzed by GraphPad Prism 6.0 statistical software and data was expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Turkey's test was used to compare the differences between means. $P<0.05$ was considered to be statistically significant.

Supplemental References

JIANG, H., SONG, N., XU, H., ZHANG, S., WANG, J. & XIE, J. 2010. Up-regulation of divalent

metal transporter 1 in 6-hydroxydopamine intoxication is IRE/IRP dependent. Cell Res, 20, 345-56.